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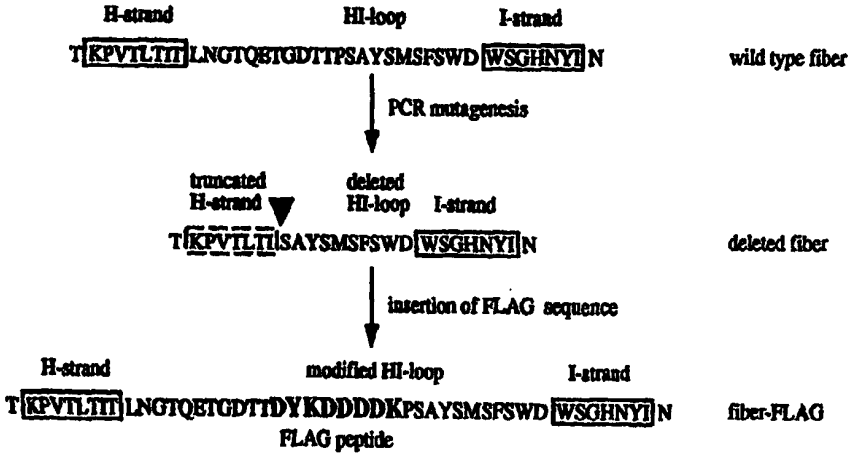
(54) Title: METHOD FOR THE PURIFICATION OF VIRUS



(57) Abstract

The utility of current recombinant adenovirus vectors for gene therapy applications is improved by designing targeted vectors capable of gene delivery to selected cell types *in vivo*. In order to achieve such targeting, incorporation of ligands in the adenoviral fiber protein, in which the protein mediates primary binding of adenovirus to its cell surface receptor, utilizes the HI loop of the fiber knob as a convenient locale for incorporation of heterologous ligands. Recombinant fiber proteins expressed in a variety of cells including baculovirus–infected insect cells and *E. coli* to demonstrate that the incorporation of the FLAG octapeptide into the HI loop does not ablate fiber trimerization and does not disturb formation of the cell–binding site localized in the knob. A recombinant adenovirus of the instant invention having this modified fiber shows that a short peptide sequence engineered in the knob is compatible with the biological functions of the fiber. A peptide incorporated into the knob according to the invention remains available for binding in the context of mature virions containing modified fibers. The invention incorporates heterologous ligands into the HI loop of the fiber knob and the properties of this locale are consistent with its employment in adenovirus retargeting strategies.

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METHOD FOR THE PURIFICATION OF VIRUS

Field of the Invention

The instant invention relates generally to vectors and methods for gene therapy, and more particularly, to an adenovirus vector containing a heterologous peptide epitope in the HI loop of the vector fiber knob and methods for purifying such a vector for application in transgene delivery.

Background of the Invention

Viruses, particularly adenoviruses have significant use as vectors for carrying genetic material into cells. Presently, adenoviral vectors are widely used in experimental systems, and it is anticipated that they will soon have a significant use in a number of genetic therapies, both in humans and animals. In order to increase the efficiency of such viral-based genetic therapies, and to minimize the side effects resultant therefrom, the viral vectors must be concentrated and/or purified.

Recombinant adenovirus vectors have found wide employment for a number of gene therapy applications (21, 35, 38). This fact has derived principally from the high levels of gene transfer achievable with this vector approach both *in vitro* and *in vivo*. Indeed, recombinant adenovirus vectors are distinguished from other available systems by their unique ability to accomplish *in situ* gene delivery to differentiated target cells in a variety of organ contexts (5, 6, 9, 10, 12, 20, 25, 27, 29, 31). Despite this property, specific aspects of the adenovirus biology have prevented the full realization of the potential of such vectors. In this regard, the broad tropism profile of the parent virus for cells of diverse tissues potentially allows unrestricted gene delivery. Thus, for the many gene therapy applications requiring targeted, cell-specific gene delivery, the promiscuous tropism of the adenovirus vector represents a confounding factor. Based on this concept,

strategies to modify the native tropism of adenovirus have been developed to allow the derivation of vectors capable of targeted gene delivery.

Strategies to achieve this end are directed at modifying specific steps in the adenoviral infection pathway. Adenoviruses of serotypes 2 and 5 normally achieve initial recognition and binding to target cells by means of interactions between the carboxy-terminal knob domain of the fiber protein and the primary receptor (4, 17, 36). After binding, RGD motifs in the penton base interact with cellular integrins of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ types (1-3, 37, 39, 40). This interaction triggers cellular internalization whereby the virions achieve localization within the endosome. Acidification of the endosome elicits conformational changes in capsid proteins, allowing their interaction with the endosome membrane in a manner that achieves vesicle disruption and particle escape. Following endosomolysis, the virion translocates to the nucleus, where the subsequent steps of the viral life cycle occur. This understanding of the key role played by capsid proteins in the viral infectious pathway has suggested strategies to alter this process via modifications of these proteins.

In this regard, genetic retargeting of adenovirus vectors via modification of viral genes encoding coat proteins, offers a simple way to achieve a significant improvement in the present generation of these gene delivery vehicles. To this end, several groups have reported genetic modifications to the knob domain of adenovirus fiber protein and incorporation of such chimeric fibers into virions. For instance, workers in the art (35, 24) reported successful generation of Ad5 virions containing fibers consisting of the tail and shaft domains of Ad5 fiber and the knob domain of Ad3, respectively. In addition, Michael et al. (30) demonstrated the incorporation of the gastrin-releasing peptide into the carboxy terminus of recombinant Ad5 fiber. This finding was extended by Legrand

et al. (30a) who achieved rescue of recombinant adenovirus vectors containing such fibers. Another report published by Wickham et al. (41) described the generation of recombinant virus containing fibers with carboxy-terminal polylysine sequences. These studies have established key feasibility issues with respect to this genetic approach but have also demonstrated a number of potentially limiting factors.

Of note, all the modifications of adenovirus fiber reported so far were directed towards the carboxy terminus of the protein. In addition, these efforts were initiated without prior knowledge of the three-dimensional (3D) structure of the fiber knob. Thus, the employment of the carboxy terminus of the fiber represented a choice not fully incorporating all relevant considerations. Clearly, 3D structural information has important bearing upon the placement of heterologous protein sequences within the knob for targeting purposes. In this regard, such localization of targeting ligands would ideally be achieved in a manner to allow their surface presentation and to minimally perturb the fiber quaternary structure. Thus, the recent crystallization of the fiber knob by Xia et al. (43, 44) has provided a level of structural resolution potentially allowing such a rational modification of the fiber protein. According to the proposed 3D model of the knob, the HI loop possesses a number of features which predict its utility as an alternative site for ligand incorporation. Specifically, the HI loop does not contribute to intramolecular interactions in the knob, therefore incorporation of additional protein sequence should not affect the trimerization of the fiber. In addition, the loop consists mostly of hydrophilic amino acid residues and is exposed outside the knob. It thus potentially demonstrates a high degree of flexibility, creating an optimal environment for ligand incorporation. Furthermore, the lengths of HI loops vary significantly in knobs of different adenovirus serotypes. This fact suggests that alterations of the original structure of the loop, such

as insertions and deletions, should be compatible with the correct folding of the entire knob domain. Finally, the HI loop is not involved in the formation of the putative cell-binding site localized in the knob.

Summary of the Invention

Based on these considerations, the instant invention develops a novel approach to modifying the adenovirus fiber protein by employing the HI loop of the knob for this purpose. This invention shows that it is possible to incorporate heterologous amino acid sequences into the HI loop without affecting the correct folding of the fiber polypeptide and its biological functions. Further, this locale offers advantages for strategies designed to achieve tropism modification based upon genetic alteration of capsid proteins.

Presently employed techniques for the purification of adenoviral vectors are based upon density centrifugation. This is an expensive and time consuming process. If adenoviral vectors are to be produced in commercially significant quantities, there is needed a low cost, rapid and simple method for their purification and concentration. Such techniques should be compatible with presently employed manufacturing processes, and should not require the use of complicated and/or expensive equipment.

Brief Description of the Drawings

Fig. 1 shows modifications of the HI loop of the fiber knob. PCR-based mutagenesis was employed to delete a portion of the fiber gene encoding the hypervariable region of the HI loop. A unique *EcoRV* restriction site was incorporated in place of the deletion to allow the cloning of segments of DNA coding for heterologous protein sequences. In the fiber-FLAG protein, deleted amino acids of the HI loop were restored, and FLAG octapeptide was incorporated between threonine-546 and proline-547. The site of deletion is indicated by a filled triangle.

Fig. 2 shows analysis of recombinant fiber proteins by polyacrylamide gel electrophoresis. Fiber proteins expressed in insect cells were analyzed by gel electrophoresis to confirm their trimeric configurations. To dissociate trimers to monomers, the proteins were denatured by boiling them in the sample buffer prior to loading them on a 7.5% polyacrylamide gel. The bands were visualized by Coomassie blue staining. (A). Six-histidine-tagged fiber proteins purified on an Ni-NTA-Sepharose column. Lane 1, wild type fiber, boiled; lane 2, wild type fiber, unboiled; lane 3, fiber-FLAG, boiled; lane 4, fiber-FLAG, unboiled; lane M, broad-range protein standards. (B). Fiber-FLAG protein purified by immunoprecipitation with anti-FLAG M2 affinity gel. Lane 1, unboiled protein; lane 2, boiled protein; lane M, broad-range protein standards. The numbers on the left indicate molecular masses of marker proteins in kilodaltons.

Fig. 3 shows inhibition of adenovirus infectivity by recombinant fiber proteins. HeLa cells were preincubated with either the wild-type (wt) fiber (A) or fiber-FLAG (B) at the indicated concentrations for 10 min at room temperature. AdCMVLuc was then added at a multiplicity of infection of 10, and incubation was continued for another 30 min at room temperature. The unbound virus was aspirated, complete medium was added, and the cells were transferred to 37°C. After 30 h the cells were lysed and luciferase activity was determined. Luciferase activities are given as percentages of the activity in the absence of blocking fiber protein. Each point represents the mean of four determinations obtained in one experiment.

Fig. 4 shows generation of Ad5F_{HI}FLAG. The master plasmid, pTG3602, was modified to incorporate a unique *SwaI* restriction site in the fiber gene, thereby creating plasmid pVK50, suitable for fiber modifications. The genome of Ad5F_{HI}FLAG was

generated by homologous DNA recombination in *E. coli* between the DNA fragment containing the fiber-FLAG gene and plasmid pVK50 linearized by *SwaI* digestion. To rescue the virus, the resulting plasmid, pVK300, which contains the complete adenoviral genome with a modified fiber gene, was cleaved with *PacI* and was then used to transfect 293 cells.

Fig. 5 shows adenovirus binding assay results. Aliquots of A549 cells containing 10^5 cells per sample were incubated for 1 h at 4°C with serial dilutions of either wild-type (wt) Ad5 fiber or fiber-FLAG (see Materials and Methods). Virions of Ad5CMVLacZ (A) and Ad5F_{HI}FLAG (B) labeled with ^{125}I were added to samples, and incubation was continued for an additional hour. The cells were washed with 4 ml of PBS containing 0.1% BSA and pelleted by low-speed centrifugation. Radioactivities of samples were determined with a gamma counter. Each point represents the mean of two determinations obtained in one experiment.

Fig. 6 shows accessibility of the FLAG peptide in the context of intact Ad5F_{HI}FLAG virions. Virions of Ad5F_{HI}FLAG purified on a CsCl gradient were dialyzed, immunoprecipitated with anti-FLAG M2-affinity gel as described herein, and eluted from the gel with free FLAG peptide. Recombinant adenovirus vector Ad5CMVLuc containing unmodified fiber was used as a negative control for binding. Aliquots of all the fractions collected throughout the purification procedure were treated with DNase I to digest traces of the cellular DNA and then treated with SDS, EDTA, and proteinase K to release adenovirus DNA from the virions. The samples obtained were analyzed on a 0.8% agarose gel, and DNA was detected by ethidium bromide staining. Lanes 1 through 3, AdCMVLuc in the supernatant containing unbound material, buffer wash, and FLAG-eluate, respectively; lanes 4 through 6, Ad5F_{HI}FLAG in the supernatant,

buffer wash, and FLAG-eluate, respectively. Lane M, DNA molecular weight standards (the bands corresponding to marker fragments ranging from 3 to 12 kb are seen on the gel).

Description of the Invention

The capsid of a gene therapy vector plays a significant role in targeting the type of host cell a vector will transfect. Modification regions exposed to the vector environment serves to direct a vector towards receptors specific to a well defined host cell population. Furthermore, such a modification also serves as a molecular recognition site for purification of modified vectors from a mixed population of vectors. While the invention is described in reference to adenovirus capsid protein modification, to incorporate a heterologous epitope into the protein in order to ablate the inherent tropism of the virus, it is appreciated that the methods, protocols and reagents herein are broadly applicable to protein coated gene therapy vectors. Protein coated gene therapy vectors are defined herein to include viruses, virions, amd mono- and bi-layer micelles having proteins attached thereto.

Despite the numerous attempts which have been made to improve adenovirus as a vector for gene therapy applications, it still suffers from a number of important disadvantages, one of them being the promiscuous tropism of this virus. Genetic modification of adenovirus coat proteins to target novel cell surface receptors is the most radical and, if successful, potentially the most efficient way to overcome this limitation. In this regard, fiber, penton base, and hexon proteins are candidates for such genetic modifications. While modifications of the penton base (42, 46) and the hexon (8, 11) have been reported, these alterations were limited to the introduction of short peptide sequences into the exterior domains of these components of the adenovirus virion. In

contrast, a larger number of studies have attempted functional modifications of the fiber protein. These attempts to modify the fiber protein have an obvious explanation: in contrast to the hexon and penton base proteins, the fiber protein mediates the primary interaction of the virus with its cognate cellular receptor and therefore dictates the tropism of the virus. In addition, due to its rod-like structure, the fiber can optimally expose a novel binding ligand engineered into its structure, thus providing efficient binding to an alternative cellular receptor. Thus, alterations to the carboxy-terminal knob domain of the fiber normally containing the cell binding site is a logical approach to modifying viral tropism.

Since the time this idea was originally employed (31), several groups of investigators have proved its utility. To this end, recombinant adenoviruses containing chimeric Ad5-Ad3 (25, 37) fiber were derived, demonstrating the possibility of creating functional fiber chimeras. In addition, it was shown that by replacing the knob domain of the fiber one can alter the receptor specificity of the virus. Furthermore, in an elegant study by Wickham et al. (45), addition of a carboxy-terminal polylysine sequence to the fiber polypeptide resulted in expanded tropism of the adenovirus vector. However, it should be noted that none of these efforts has addressed the goal of ablating the native tropism of the adenovirus vector; in these approaches, novel tropism distinct from the pre-existing natural tropism of the vector was engineered.

Until recently, the ability to accomplish the practical design of retargeted adenovirus vectors was limited by two major problems: lack of knowledge of the structure of the fiber knob domain and difficulty in manipulating the fiber gene in the context of the adenovirus genome. In this regard, publication of the 3D model of the Ad5 fiber knob by Xia et al. (47, 48) and the development of a novel genetic method by

Chartier et al. (7), which allow modification of virtually any region of the adenovirus genome, will dramatically facilitate efforts to retarget the adenovirus via alterations to the knob domain of the fiber. The instant invention derives from adenovirus vectors with modified fibers containing novel peptide ligands. Herein is described the utilization of the HI loop of the fiber knob as an alternative site for incorporation of heterologous peptide sequences. According to the 3D model of the Ad5 fiber knob, the HI loop does not contribute to interactions within the knob which stabilize its trimeric configuration and is not involved in the formation of the receptor binding site. Importantly, due to the prevalence of hydrophilic amino acid residues in its primary sequence, the HI loop is exposed outside the knob, thereby facilitating the interaction of potential ligand with the cellular receptor.

For initial proof of the concept, a FLAG coding sequence was incorporated into the region of the fiber gene corresponding to the HI loop and expressed this modified gene in baculovirus infected insect cells. An amino terminal six-His tag incorporated into the design was used for simple chromatographic purification of recombinant fiber protein. Baculovirus-directed expression of this recombinant full size fiber was efficient, and according to our gel analysis and ELISA with the trimer-specific anti-fiber monoclonal antibody MAb, the product of expression was trimeric.

Previous success with bacterial expression of function and trimeric Ad3 and Ad5 fiber knobs (16, 21, 23, 31) could not be reproduced in attempts to generate modified knobs in *E. coli*. This also corresponds to the results obtained with bacterial expression of modified fiber knobs by other investigators (M. Mehtali, personal communication), suggesting that the adenovirus fiber knobs express in *E. coli* have limited utility and cannot be used as a molecular model for modifications of this domain of the fiber protein.

To further characterize the fiber-FLAG protein produced in insect cells, the accessibility of FLAG in the context of the fiber trimer was demonstrated. For this an assay was employed based on the specific interaction of FLAG-tagged proteins with M2-affinity gel containing anti-FLAG monoclonal antibody. This analysis confirmed that the FLAG peptide is localized on the surface of the trimeric knob and is available for binding, thereby supporting the hypothesis about surface localization of the HI loop. By employing the fiber-FLAG chimera to block adenovirus infection it is shown that insertion of the FLAG peptide into the HI loop of the knob does not affect the correct folding of the cell binding domain localized in the knob. This is a significant finding considering that the HI loop connects β-strands H and I, which are hypothesized to be involved in binding to the cellular receptor (38, 47, 48).

To incorporate fiber-FLAG chimeras into the adenovirus virion, a recombinant adenovirus genome was generated by using a novel method described recently (7). To reach this end, a master plasmid, pTG3602, from Transgene was modified to engineer a vector which greatly facilitates modifications of the fiber gene in the adenovirus genome. By using this plasmid, it was possible to generate recombinant genome and rescued the virus of interest, Ad5F_{HI}FLAG. Importantly, this new virus is produced in high yields and demonstrates dynamics of infection identical to those of the wild type Ad5. Successful rescue of Ad5F_{HI}FLAG, as well as subsequent characterization of the virion, confirmed our conclusions based on the results obtained with fiber-FLAG protein expressed in baculovirus-infected insect cells, thereby making baculovirus an expression system of choice for further fiber-modeling experiments.

Thus, it is shown that the HI loop of the fiber knob is a convenient site for incorporation of heterologous peptide ligands which may be successfully utilized in order

to target adenovirus vectors for gene therapy applications. This location in the knob can be used either as an alternative site or in addition to carboxy-terminal modifications of the fiber protein, offering a unique loop-like environment, which may be required for proper biological functioning of some ligand sequences. For example, this structure may be beneficial for peptide ligands obtained from phage display libraries containing random peptide sequences flanked with two cysteine residues forming a disulfide bridge (23, 24). In addition, ligands with the loop-like configuration may be less susceptible to degradation by cellular carboxypeptidases than ligands positioned at the carboxy terminus of the fiber. To realize the full potential of the HI loop for ligand incorporation, recombinant adenoviruses containing different targeting moieties in this locale are created. Generation of recombinant adenoviruses containing fibers with targeting ligands incorporated into the HI loop of the knob will facilitate further efforts towards an improved adenovirus vector for gene therapy applications. The successful use of the FLAG epitope in binding experiments suggests that this or a similar purification tag can be incorporated into an adenovirus virion to facilitate its purification. This simple purification technique occurs at atmospheric laboratory pressure and does not require expensive laboratory equipment such as ultracentrifuges or high pressure liquid chromatography systems and can be easily scaled up if needed.

The present invention, provides a method for purifying and concentrating virus, and the method is particularly suited for use with adenoviral vectors. In accord with the method of the present invention, as will be described in further detail hereinbelow, an adenoviral vector is modified so that its protein capsid expresses one or more heterologous epitopes thereupon. These foreign epitopes distinguish the modified adenovirus from the corresponding, unmodified virus and provide an active site which

is capable of binding to an antibody, chelating agent, other chemical reagent or the like. In accord with present invention, the modified virus is contacted with an appropriate binding reagent, which itself is preferably immobilized, as for example in a chromatography column. The modified virus is then bound and retained, and may be collected by freeing from the agent.

For example, an appropriate binding reagent may be immobilized on a support medium in a column, and process liquid including the virus may then be flushed through the column. The column will selectively bind the modified virus, while permitting the unmodified virus, and other materials to pass therethrough. The modified virus may then be eluted from the column by flushing with an appropriate reagent. In this manner, a purified, concentrated sample of the adenoviral vector is obtained.

The protein capsid of the virus is preferably modified by utilizing a plasmid to insert genetic information into the adenovirus to cause it to express the heterologous epitope thereupon. In some instances, modification of the capsid will be at a carboxy terminal of the capsid fibers. In other instances, the modification will take place at the knob domain of the fiber; most preferably at the H-I loop of the knob domain.

In one particular series of experiments, a plasmid vector designated pTG3602S is utilized to generate recombinant adenoviral genomes which contain genes encoding: fiber-short linker-RGS6H; fiber-short linker-somatostatin; in the capsids of adenovirus. This sequence was expressed at the C-terminus of the capsid fiber of an adenovirus designated Ad5FcRGS6H. The presence of the modified fiber gene in the viral genome was confirmed by a cycle sequencing readout on viral DNA isolated from purified virions. The presence of C-terminal modifications in the fibers was confirmed by Western blot analysis carried out on purified virus.

It was found that the RGS6H tag engineered into the fiber is accessible to anti-RGS4H monoclonal antibodies. This was confirmed in an experiment in which the viral lysate was loaded on a Ni-NTA column. The column was first washed with 40mM imidazole to flush unbound virus therefrom. The column was then developed with 0.5M imidazole to elute the modified virus therefrom. A different viral material, namely AdCMV-Luc was added to the column as negative control material, and treated as above. All samples from the column experiments were lysed with SDS to release viral DNA, and analyzed by agarose electrophoresis. The analysis showed that AdCMV-Luc did not adhere to the column and was washed therefrom by the 40mM imidazole, but that the Ad5FcRGS6H modified virus bound to the column and was only eluted therefrom by the 0.5M imidazole. The foregoing demonstrates that plasmids may be employed to modify the capsid of a virus to produce a heterologous epitope thereupon, and that this modified virus may be separated from other virus by use of an affinity reagent.

It is to be understood that within the general principles of this invention, the adenovirus vector may be made to selectively bind to an appropriate affinity reagent, by modifying the protein capsid thereof so as to cause the capsid to have a novel epitope thereupon. In this manner, the virus may be selectively separated from unmodified virus, and other materials. The methodology of the present invention may be employed to collect, concentrate and purify viral materials through a process which is simple, low cost and reliable. Furthermore, the process is compatible with standard techniques used to prepare adenoviral vectors. Consequently, the present invention greatly facilitates the production of materials for a variety of genetic therapies.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the instant invention.

Example 1 - Cells.

293 cells (16) obtained from Microbix (Toronto, Ontario, Canada) and HeLa human adenocarcinoma cells and A549 human lung carcinoma cells obtained from American Type Culture Collection (Rockville, Md.) were maintained in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 from Mediatech (Herndon, Va.) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, Utah) at 37°C and 5% CO_2.

Example 2 - Enzymes.

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and proteinase K were from either New England Biolabs (Beverly, Mass.) or Boehringer Mannheim (Indianapolis, Ind.).

Example 3 - Protein assay.

The concentrations of purified proteins were determined by the Bradford protein assay (Bio-Rad, Hercules, Calif.) with bovine gamma globulin as the standard.

Example 4 - Antibodies.

Anti-fiber monoclonal antibodies 4D2 (19) and 1D6.14 (14) were generated at the University of Alabama at Birmingham Hybridoma Core Facility. Anti-FLAG monoclonal antibody M2 and M2-affinity gel were purchased from Scientific Imaging Systems (Eastman Kodak, New Haven, Conn.)

Example 5 - Constructi n of recombinant plasmids.

To generate a gene encoding the Ad5 fiber knob domain with the HI loop deleted, a PCR technique was employed. Two pairs of primers F1 (5' TAAGGATCCG GTGCCATTAC AGTAGGAAAC AAAAATAA 3') and R1 (5' CATAGAGTAT GCAGATATCG TTAGTGTTAC AGGTTTAGTT TT G 3'), and F2 (5' GTAACACTAA CGATATCTGC ATACTCTATG TCATTTTCAT GG 3') and R2 (5' CCCAAGCTTA CAATTG AAAA ATAAACACGT TGAAACATAA C 3'), were used to amplify portions of the fiber gene corresponding to positions 1159 to 1451 and 1642 to 1747, respectively. In addition, the second fragment also contains 43 bp of Ad5 DNA adjacent to the 3' end of the fiber gene in the viral genome. The fragments generated were then gel purified, mixed, and joined by the third PCR using primers F1 and R2. The product obtained contains a unique *EcoRV* restriction site in place of the deleted portion of the sequence encoding the HI loop, as well as *BamHI* and *HindIII* sites inserted into the ends of the molecule to facilitate subsequent cloning. This DNA fragment was cleaved with *BamHI* and *HindIII* and cloned into the *BamHI-HindIII*-digested bacterial expression vector pQE30 (Qiagen, Santa Clara, Calif.), resulting in plasmid pQE.KNOBΔHI.

To construct an expression plasmid with the FLAG sequence incorporated into the HI loop of the fiber, oligonucleotides TACACTAAAC GGTACCCAGG AAACAGG AGA CACAACTGACT ACAAGGA CGACGATGACAAG CC and GGCTTGTCATC GTCGTCCTTG TA GTCAGTTGT GTCTCCTG TTT CCTGGGTACCG TTTAGTGTA were annealed to form a duplex and cloned into *EcoRV*-digested pQE.KNOBΔHI. The plasmid containing the duplex in the correct orientation was designated $pQE.KNOB_{HI}FLAG$.

The transfer plasmids for the generation of recombinant baculoviruses expressing chimeric fibers were made as follows: a *BglII-MfeI* fragment from pQE.KNOB$_{HI}$FLAG was utilized to replace the *BglII-MfeI* fragment in the vector pBS.F5.UTR which has been described previously (24), thereby generating pBS.F5$_{HI}$FLAG. A *BssHII-Xho*I fragment from pBS.F5$_{HI}$FLAG was then cloned into the *BssHII-XhoI*-digested baculovirus transfer vector pFastBac1 (Life Technologies, Gaithersburg, Md.), resulting in pFB.F5$_{HI}$FLAG. To introduce the six-His purification tag into the amino terminus of the chimeric fiber, the *BamHI-BssHII* fragment of pFB.F5$_{HI}$FLAG was replaced with a synthetic duplex made with oligonucleotides GATCCATGCA TCACCATCAC C ATCACAAG and CGCGCTTGTG ATGGTGATGG TGATGCATG, which encodes MetHis$_6$Lys. The resultant plasmid, pFB6H.F5$_{HI}$FLAG, contains the gene coding for a fiber with an amino-terminal six-His tag and FLAG peptide inserted into the HI loop. To derive a similar plasmid containing the fiber gene with the HI loop coding sequence unmodified, the *BssHII-MfeI* fragment in pFB6H.F5$_{HI}$FLAG was replaced with homologous fragment from pNEB.PK3.6 (24), generating pFB6H.F5.

In order to clone the gene encoding the fiber with the FLAG sequence in the HI loop into the fiber shuttle vector pNEB.PK3.6, a *BstXI-MfeI* fragment of the wild type fiber gene contained in this plasmid was replaced with a *BstXI-MfeI* fragment from pQE.KNOB$_{HI}$FLAG, thereby creating pNEB.F5$_{HI}$FLAG.

To facilitate the generation of recombinant adenovirus genomes by homologous recombination in *Escherecia coli*, plasmid pTG3602 (7), obtained from Transgene (Strasbourg, France), was engineered to create a specialized vector suitable for modifications of the fiber gene. To accomplish this end, an *NdeI* site localized in the fiber gene was employed. Plasmid pTG3602 was partially digested with *NdeI* and ligated

with an *NdeI-SwaI* linker, TACCCATTTAAATGGG. This plasmid, containing a *SwaI* site in the fiber gene was designated pVK50.

A recombinant adenovirus genome containing a gene encoding the fiber-FLAG protein was generated by homologous DNA recombination in *E. coli* BJ5183 between pVK50 linearized with *SwaI* and the 3-kb EcoRI fragment from pNEB.F5$_{HI}$FLAG containing the gene of interest, as described by Chartier et al. (7). The newly generated genome was then excised from the resultant plasmid, pVK300, and employed to rescue the virus.

Example 6 - Viruses.

Ad5 vectors, AdCMVLuc and AdCMVLacZ, which express firefly luciferase and bacterial β-galactosidase (18), respectively, were obtained from R. D. Gerard, the University of Texas Southwestern Medical Center, Dallas, Tex.

Ad5F$_{HI}$FLAG was generated by transfection of 293 cells with *PacI*-digested pVK300, as previously described (7).

Adenoviruses were propagated on 293 cells and purified by centrifugation in CsCl gradients according to a standard protocol (15). Determination of virus particle titer was accomplished spectrophotometrically by the method described by Maizel et al. (28), with a conversion factor of 1.1 x 10^{12} viral particles per absorbance unit at 260 nm. To determine the titer of infectious viral particles on 293 cells, a plaque assay was employed as described by Mittereder et al. (32).

Recombinant baculoviruses expressing chimeric fibers were generated with a Bac-to-Bac expression kit from Gibco-BRL (Life Technologies) according to the manufacturer's protocol.

Example 7 - Expressi n and purification f six-His-tagged recombinant proteins.

Recombinant fibers were expressed in *Spodoptera frugiperda* Sf9 cells infected with recombinant baculovirus by the method recommended for the Bac-to-Bac system (Life Technologies). Recombinant proteins were then purified by immobilized metal ion affinity chromatography on Ni-nitrilotriacetic acid (NTA)-Sepharose (Qiagen) by following recommendations from the manufacturer.

Example 8 - ELISA.

In order to characterize recombinant fiber proteins, an enzyme-linked immunosorbent assay (ELISA) was employed. The six-His tagged fibers were immobilized on Ni-NTA HisSorb Strips (Qiagen) essentially as described in the Qiagen manual. 200 μl of fiber protein solution at a concentration 1 μg/ml was added to each well of an Ni-NTA HisSorb Strip and incubated for 1 h at room temperature. After incubation the wells were washed four times with phosphate-buffered saline (PBS)-Tween buffer, and 200 μl of anti-fiber antibody (1:2000 dilution) or anti-FLAG antibody (1:140 dilution) was added. Following incubation at room temperature for 2 h the wells were washed again and incubated with 1:10,000 dilution of goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (HRP) for 45 min. The wells were then washed four times with PBS-Tween buffer and developed with 2',2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (diammonium salt). The ABTS-HRP reaction was read in a microtiter plate reader set at 405 nm.

Example 9 - FLAG accessibility assay.

To demonstrate the binding of the FLAG-tagged fiber protein incorporated into intact virions of $Ad5F_{HI}FLAG$ to anti-FLAG M2 monoclonal antibody, an immunoprecipitation assay was employed. $Ad5F_{HI}FLAG$ or AdCMVLuc purified on CsCl gradients were dialyzed against HEPES buffer (10 mM HEPES, 1 mM $MgCl_2$, 10% glycerol, [pH 7.4]) and absorbed onto M2-affinity gel (Eastman Kodak) as follows. Fifty microliters of dialyzed virus containing 10^{11} viral particles was mixed with 100 μl of M2-affinity gel equilibrated with HEPES buffer containing 50 mM NaCl and 0.5% bovine serum albumin (BSA) and then incubated overnight at 4°C on a rotating wheel. Following incubation, the gel was spun down by brief centrifugation in a microcentrifuge. The supernatant was collected for further analysis and the gel was washed with 0.5 ml of Tris-buffered saline (TBS). Virus was eluted at 4°C with 50 μl TBS containing 400μg of FLAG peptide per ml. The supernatant containing unbound material, the wash, and the eluate were then employed to detect the presence of the virus. For this, aliquots of these fractions were treated for 1 h at 37°C with sodium dodecyl sulfate, EDTA, and proteinase K at final concentrations of 1%, 10 mM and 100 μg/ml, respectively. The samples were analyzed by agarose gel electrophoresis to detect viral DNA.

Example 10 - Purification of the fiber-FLAG protein by immunoprecipitation.

The recombinant fiber-FLAG protein was expressed in baculovirus infected Sf9 cells as follows. For large scale expression of the fiber-FLAG protein, monolayers of Sf9 cells in T75 flasks were infected with recombinant baculovirus at multiplicity of infection of 5 to 10 and then were incubated at 28°C until a complete cytopathic effect was

observed. At 2 to 3 days postinfection, the cells were scraped, pelleted by low speed centrifugation, and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml). The cells were then incubated on ice for 30 min. The lysate was cleared by centrifugation at 12,000 x g for 5 minutes in a microcentrifuge. The cleared lysate was mixed with the slurry of M2 affinity gel, and the rest of the procedure was performed as described above for immunoprecipitation of $Ad5F_{HI}FLAG$.

Example 11 - Trimerization assay of recombinant proteins.

To determine whether the fiber proteins expressed in baculovirus infected insect cells could form trimers, these proteins were analyzed by SDS-polyacrylamide gel electrophoresis as previously described (30). Proteins were either boiled prior to electrophoresis, to dissociate the trimers, or loaded on the gel without denaturation. The trimeric or monomeric configuration of these molecules was thus determined based on their mobilities in the gel.

Example 12 - Inhibition of virus mediated gene transfer by recombinant fiber proteins.

The ability of the fiber-FLAG chimera to block adenovirus-mediated gene transfer was evaluated in infection inhibition experiments similar to those described previously (17, 24, 34, 36). Briefly, monolayers of HeLa cells grown in a 24-well plate were preincubated at room temperature with serial 10-fold dilutions of either wild type fiber or fiber-FLAG protein prior to infection with a replication defective recombinant adenovirus expressing firefly luciferase, AdCMVLuc. Unbound virus was washed, and the cells were incubated at 37°C to allow internalization of AdCMVLuc and expression

of the luciferase gene. A luciferase assay of the lysates of infected cells was performed 30 h postinfection with a luciferase assay system from Promega (Madison, Wis.).

Example 13 - Virus binding assay.

Human lung carcinoma A549 cells grown in T75 flasks and then harvested with EDTA, washed once with PBS, pelleted and resuspended to a final concentration of 10^7 cells/ml in DMEM-Ad medium (DMEM, 20mM Hepes, 0.5%BSA) as described by Wickham et al (45). One-hundred-microliter aliquots of the cells were transferred to 5 ml test tubes and incubated for 1 h at 4°C with 100 μl of recombinant fiber diluted in DMEM-Ad medium.

The recombinant adenoviruses AdCMVlacZ and Ad5F_{HI}FLAG were purified on a CsCl gradient and dialyzed against buffer containing 10 mM HEPES, 1 mM MgCl2, 10% glycerol [pH 7.4]. Aliquots of both viruses containing 50 μg of viral protein were labeled with ^{125}I using IODO-BEADS iodination reagent (Pierce, Rockford, Ill.) as previously described (20). Labeled viruses were purified from unincorporated ^{125}I by gel filtration on PD-10 columns (Pharmacia, Piscataway, N.J.). Fifty-microliter aliquots of labeled virions with total radioactivities of 10^5 cpm were then added to A549 cells preincubated with fiber dilutions or PBS and incubated at 4°C for another hour.

The samples were diluted with 4 ml of PBS containing 0.1% BSA, and the cells were pelleted by centrifugation. Supernatant containing unbound virus was aspirated, and the radioactivities of cell pellets were determined in a gamma counter.

Example 14 - Expression of recombinant fiber knobs in *E. coli*.

To test the concept of the suitability of the HI-loop of the fiber knob for incorporation of heterologous protein sequences, recombinant knob proteins expressed in *E. coli* were employed. Previously such recombinant knobs produced by bacterial cells were successfully utilized for a variety of applications (Gerard, McCleland, Louis, Krasnykh). To achieve this aim, a PCR approach was used to derive a gene encoding Ad5 fiber knob with a partial deletion in the HI-loop. This deletion was engineered to remove amino acids TLNGTQETGDTTP from the HI-loop of the knob, and to introduce unique EcoRV site in place of deleted sequence, thereby facilitating the cloning of alternative sequences in this region (Figure 1). The deletion made removed the portion of the HI-loop which varies most significantly in the fiber knobs of different serotypes of human adenoviruses. The sequence generated by PCR contained an open reading frame corresponding to two segments of the fiber protein including amino acids glycine-387 through isoleucine-534 and serine-548 through glutamine-581 (here and in the following text the coordinates are given according to the wild type Ad5 fiber protein sequence). This sequence was cloned into bacterial expression vector, pQE30, thereby creating a fusion gene encoding the deleted fiber knob and containing amino terminal 6His purification tag. The newly generated plasmid, pQE.KNOBΔHI, was then utilized as a cloning vector to incorporate fragments of DNA encoding cleavage sites for highly specific proteolytic enzymes: Factor Xa, thrombin and enterokinase. These recognition sequences were chosen as probes to evaluate the accessibility of the selected portion of the HI-loop. Therefore, using pQE.KNOBΔHI expression plasmids pQE.KNOB$_{HI}$F-Xa, pQE.KNOB$_{HI}$THR and pQE.KNOB$_{HI}$FLAG, containing genes were generated which encoded knobs with cleavage sites for Factor Xa, thrombin and enterokinase,

respectively. By incorporating these sequences in the ORF of the knob the previously deleted codons were restored. Therefore in the final constructs the cleavage sequences for aforementioned proteolytic enzymes were introduced as insertions between threonine-546 and proline-547. These expression plasmids were then utilized to direct expression of recombinant knob proteins in *E. coli* cells.

In contrast to the wild type Ad5 knob used in our previous study ((21)), all recombinant knobs with insertions of proteolysis sites in the HI-loop, as well as the one with a partially deleted loop sequence, showed very low levels of expression corresponding to less than 5% of the yield of the wild type knob. Moreover, with the exception of the knob containing Factor Xa cleavage site, these knobs failed to trimerize (data not shown). Preparations of the knob with Factor Xa recognition sequence contained trimers in the amount of 95% of total protein purified, the rest being monomeric molecules. However, these trimers were gradually converting to monomers with the half life of 7 days when stored at 4°C, making it difficult to use this recombinant protein for subsequent experiments. In addition, none of the proteins produced was susceptible to the cleavage with relevant protease under a variety of different conditions.

These data have demonstrated that recombinant Ad5 fiber knob expressed in *E. coli* cells is not an appropriate model for experiments involving modifications of its amino acid sequence.

Example 15 - Characterization of recombinant fibers expressed in baculovirus infected insect cells.

To test the concept of the suitability of the HI loop of the fiber knob for incorporation of heterologous protein sequences, we first employed recombinant fiber proteins expressed in a baculovirus expression system. This system has already proved

its utility for the expression of functional Ad2, Ad3, and Ad5 fiber proteins, as well as Ad3-Ad5 and Ad5-Ad3 fiber chimeras (13, 26, 33, 37).

To achieve this aim, a PCR approach was used to derive a gene encoding the Ad5 fiber knob with a partial deletion in the HI loop. This deletion was engineered to remove amino acids TLNGTQETGDTTP from the HI loop of the fiber knob domain and to introduce a unique *EcoRV* site in place of the deleted sequence, thereby facilitating the cloning of alternative sequences in this region (Fig. 1). The deletion removed the portion of the HI loop which varies most significantly in the fiber knobs of different serotypes of human adenoviruses. The sequence generated by PCR contained an open reading frame corresponding to two segments of the fiber protein including amino acids glycine-387 through isoleucine-534 and serine-548 through glutamine-581 (coordinates given are according to those of the wild type Ad5 fiber protein sequence). This sequence was cloned into the plasmid vector pQE30. The newly generated plasmid, pQE.KNOBΔHI, was then utilized as a cloning vector to incorporate a fragment of DNA encoding the FLAG octapeptide (DYKDDDDK), which has been widely used as a detection and purification tag in a variety of studies. Thus, this FLAG peptide was chosen in fiber constructs as a probe to determine whether a heterologous peptide sequence incorporated into the HI loop of the knob was accessible in the context of a trimeric fiber molecule. By incorporating this sequence into the open reading frame of the knob, the previously deleted condons were restored. Therefore, in the newly generated plasmid, pQE.KNOB$_{HI}$FLAG, the FLAG coding sequence was introduced as insertions between threonine-546 and proline-547. This plasmid was then employed to construct a full-size recombinant fiber gene in a baculovirus transfer vector. A similar transfer plasmid containing the wild type fiber gene was designed for control purposes.

In order to facilitate subsequent purification of the expression products, we introduced into the designs of both genes a sequence encoding an amino-terminal six-His tag. These plasmids were then utilized to generate two recombinant baculoviruses containing fiber genes encoding wild type Ad5 fiber and a fiber protein containing FLAG peptide in the HI loop of the knob domain.

Recombinant fibers were recovered from the lysates of baculovirus-infected insect cells with Ni-NTA-Sepharose designed for purification of the six-His-tagged proteins. The yield of purified fibers was in the range of 10 μg of protein per 10^6 infected cells. Analysis by SDS-polyacrylamide gel electrophoresis of both recombinant proteins showed that they formed stable trimers which, when boiled in the gel loading buffer, dissociated into monomers of the expected molecular mass of 63 kDa (Fig. 2A). This result demonstrated that the incorporation of a short peptide sequence in the HI loop of the knob does not ablate trimerization of the fiber. Therefore, by using the baculovirus expression system we were able to obtain preparative amounts of the recombinant fibers of interest which were suitable for subsequent assays.

Example 16 - Accessibility of the FLAG peptide in the context of trimeric fiber.

To find out whether the FLAG peptide introduced into the HI loop of the fiber was available for binding, an assay was used based on the specific interaction of the FLAG-tagged proteins with an affinity matrix containing anti-FLAG monoclonal antibody. For these experiments, the recombinant fiber protein with the FLAG sequence in the HI loop (fiber-FLAG) was purified on an Ni-NTA-Sepharose column and then immunoprecipitated with M2-affinity gel. Protein bound to the matrix was then specifically eluted with FLAG peptide and analyzed on an SDS containing

polyacrylamide gel (Fig. 2B). According to this analysis, the fiber-FLAG protein efficiently bound to M2-affinity gel, demonstrating the availability of the FLAG epitope for interaction with an anti-FLAG monoclonal antibody in the context of the trimeric fiber molecule. Importantly, this interaction did not affect the stability of the trimer, suggesting that a recombinant virion containing a novel ligand incorporated in the HI loop of the fiber knob will maintain its structural integrity throughout the binding step of the infection.

Example 17 - Inhibition of adenovirus infection by recombinant fiber-FLAG protein.

A fiber-FLAG recombinant protein was employed to block adenovirus infection in the in vitro setting. This established assay is based on the fact that recombinant adenovirus fiber proteins are capable of blocking infection by the adenovirus from which they were derived. In addition, this inhibition of viral infection takes place in a dose dependent manner.

In experiments, HeLa cells seeded in 12-well tissue culture plates were preincubated with various concentrations of the wild-type Ad5 fiber or fiber-FLAG protein prior to infection with the recombinant Ad5 vector AdCMVLuc, which expresses firefly luciferase as a reporter. Previously, it was shown that this assay, based on gene transfer by the viral vector, generates data correlating well with a classic binding assay accomplished with radiolabeled virus (24). Thirty hours postinfection, the cells were lysed and the lysates were utilized for the luciferase activity assay (Fig. 3). According to this assay, both fiber proteins blocked infection by AdCMVLuc in a dose dependent manner and demonstrated identical profiles of infection inhibition. These data confirmed that incorporation of heterologous peptide sequences into the HI loop of the fiber knob

does not affect the correct folding of the cell-binding site formed by the carboxy-terminal portion of the fiber protein.

Example 18 - Characterization of the fiber-FLAG protein by ELISA.

To obtain additional evidence supporting the functional utility of the fiber-FLAG protein, this recombinant protein by ELISA was analyzed, employing several monoclonal antibodies specific for the FLAG epitope and different conformations of the Ad5 fiber. To achieve this end, wild type fiber and fiber-FLAG proteins expressed in insect cells were absorbed on HisSorb ELISA strips covered with Ni-NTA (Qiagen) and probed with anti-fiber antibody 4D2 or 1D6.14 or anti-FLAG antibody M2. Antibody 4D2 reacts with Ad5 fiber monomers and trimers and was used in this assay as a positive control, whereas antibody 1D6.14 binds to an as yet unidentified conformational epitope in the fiber knob and is trimer specific. The ELISA strips were then developed with goat anti-mouse antibody-HRP conjugate.

As seen in Table 1, both fiber proteins efficiently reacted with anti-fiber antibodies 4D2 and 1D6.14, thereby suggesting that the 3D structure of the knob in the fiber-FLAG molecule is identical to that of the wild type fiber. In addition, the fiber-FLAG chimera specifically reacted with anti-FLAG antibody M2, confirming the availability of this epitope for binding in the context of a trimeric fiber molecule. Thus, these results validated the data generated earlier by gel electrophoresis analysis of Ni-NTA- or M2-affinity gel purified fiber-FLAG protein, providing the rationale for the incorporation of the fiber-FLAG chimera into the adenovirus virion for further characterization.

Example 19 - Generati n of Ad5F$_{HI}$FLAG.

Despite the fact that the data obtained with the recombinant fiber-FLAG protein supported the concept of its functional utility in the context of the adenovirus virion, only successful generation of the recombinant virus would prove our hypothesis regarding the compatibility of the modifications of the HI loop of the fiber knob with viral functions. Therefore, the task of incorporating the fiber-FLAG chimera into the adenovirus virion was undertaken.

In order to derive this virus, a novel genetic method based on homologous DNA recombination in *E. coli* cells was utilized (7). In brief, this method involves recombination between two linear DNA molecules cotransformed into bacterial cells to generate a recombinant adenovirus genome. One of these molecules is plasmid pTG3602, or its derivative, containing the full-size adenovirus genome cloned in the bacterial vector and flanked with two *PacI* sites. The second partner in this recombination scheme is the genetic construct of interest flanked with two segments of adenovirus genomic DNA which dictate the localization of this construct in the adenovirus genome generated as a result of the recombination. This DNA sequence can be either a transgene or the original Ad5 gene, modified by traditional methods of genetic engineering in the context of small recombinant plasmids. To reduce the nonrecombinant background generated by pTG3602, prior to transformation this plasmid was cleaved with a restriction enzyme within or near the region of the genome where the final construct was going to be inserted. Although this method has numerous advantages compared to traditional generation of recombinant adenovirus genomes by homologous recombination in mammalian cells, it requires the existence of unique restriction sites within the regions of the adenovirus genome to be modified. However, Ad5 genomic

DNA in pTG3602 does not contain any unique restriction sites in the fiber gene, which limits its utility for modifications of fiber. Thus, to overcome this limitation, this plasmid was modified by inserting a unique cleavage site for the restriction endonuclease *SwaI* into the fiber gene. To this end, one of the two *NdeI* sites present in Ad5 DNA and localized 47 bp downstream from the fiber gene's 5' end was converted into *SwaI* site by insertion of an *SwaI*-linker (Fig. 4). The plasmid generated, pVK50, was then utilized for homologous recombination with the fragment of DNA containing the gene encoding fiber-FLAG flanked with viral DNA adjacent to the fiber gene in the Ad5 genome. As a result of this recombination, a plasmid, pVK300, containing a modified fiber gene in the context of the complete adenovirus genome was derived. Adenovirus DNA was released from pVK300 by *PacI* digestion and used for transfection of 293 cells to rescue the virus as described previously (7).

DNA isolated from CsCl gradient-purified virions of the newly generated virus, $Ad5F_{HI}FLAG$, was subjected to PCR analysis and cycle sequencing to confirm the presence of the FLAG coding sequence in the fiber gene incorporated in the genome. According to both analyses, $Ad5F_{HI}FLAG$ indeed contained the fiber gene of interest.

Example 20 - Characterization of $Ad5F_{HI}FLAG$ by a cell-binding assay.

The yield of this virus grown on 293 cells, approximately 10^{11} plaque forming units (PFU) per preparation obtained from 20, 75 cm^2 tissue culture flasks, was comparable to what we normally obtain when growing the wild-type Ad5. Also, we did not see any delay in plaque formation dynamics either when rescuing the virus or when expanding it. These observations suggested that the introduction of the FLAG peptide

in the HI loop of the knob did not significantly affect the correct folding of the fiber molecule and its biological functions.

In order to prove this, radiolabeled Ad5F_{HI}FLAG was employed to investigate its ability to bind the fiber receptors on the cell surface. In this assay ^{125}I-labeled Ad5F_{HI}FLAG was allowed to bind A549 human lung carcinoma cells, which are known to express high levels of Ad5 fiber receptors. Baculovirus expressed wild type Ad5 fiber and fiber-FLAG were used as competitors to selectively block cellular receptors and inhibit virus binding. The recombinant adenovirus vector Ad5CMVLacZ containing wild type fibers was used as a control. The results of this experiment showed both viruses demonstrate identical dose responses when competing with fibers of either type. Thus, incorporation of the heterologous peptide in the HI loop of the fiber-FLAG protein did not have any negative effect on the formation of the cell binding site localized in the knob and, therefore, did not affect virus infectivity.

Example 22 - Characterization of Ad5F_{HI}FLAG by antibody binding.

Since the ultimate goal of our strategy is engineering of a targeting ligand into the knob, it was necessary to determine whether such a ligand would be available for interaction with its target cell surface receptor after incorporation into the adenoviral virion. To this end, the FLAG sequence incorporated into the fiber of Ad5F_{HI}FLAG was employed to test the accessibility of the HI-loop of the knob in a context of intact adenoviral particle. This was accomplished in an assay similar to the one used to evaluate FLAG accessibility in the recombinant fiber-FLAG protein expressed in insect cells. Virions of purified on CsCl gradient were dialyzed against Hapes buffer and incubated with M2 affinity gel to allow interaction between FLAG peptide and anti-

FLAG monoclonal antibody conjugated to the gel matrix. Similarly prepared virons of AdCMVLuc containing wild type fibers were utilized in this experiment as a negative control. After incubation the buffer containing unbound material was collected and the gel was washed with the buffer to remove the traces of free virus. Finally, the viruses were eluted from the gel by soluble FLAG peptide. Aliquots of the samples collected were treated with proteinase K to release viral DNA from virions which was then visualized by agarose gel electrophoresis (Figure 5). As expected, virons of AdCMVLuc did not react to M2 antibody and were detected only in the fraction containing unbound virus and in the wash. In marked contrast, Ad5F_{HI}FLAG particles efficiently bound to the affinity gel, since viral DNA was present exclusively in FLAG peptide eluate. Thus, establishing that heterologous ligand sequence engineered into the HI-loop of the knob domain of the fiber incorporated in intact Ad5 virion remains accessible for interaction with the relevant receptor structure, thereby providing a rationale for generation of genetically targeted adenoviral vectors on this basis.

Example 22 - FLAG accessibility in the context of the Ad5F_{HI}FLAG virion.

To insert a targeting ligand into the knob, it was necessary to determine whether such a ligand would be available for interaction with its target cell surface receptor after incorporation into the adenovirus virion. To this end, the FLAG sequence incorporated into the fiber of Ad5F_{HI}FLAG was employed to test the accessibility of the HI loop of the knob in the context of an intact adenovirus particle. This was accomplished in an assay similar to the one used to evaluate FLAG accessibility in the recombinant fiber-FLAG protein expressed in insect cells. Virions purified on a CsCl gradient were dialyzed against HEPES buffer and incubated with M2-affinity gel to allow interaction between

the FLAG peptide and an anti-FLAG monoclonal antibody conjugated to the gel matrix. Similarly prepared virions of AdCMVLuc containing wild-type fibers were utilized in this experiment as a negative control. After incubation, the buffer containing unbound material was collected and the gel was washed with the buffer to remove the traces of free virus. Finally, the viruses were eluted from the gel with soluble FLAG peptide. Aliquots of the samples collected were treated with proteinase K to release viral DNA from virions, which was then visualized by agarose gel electrophoresis (Fig. 7). As expected, virions of AdCMVLuc did not react with M2 antibody and were detected only in the fraction containing unbound virus and in the wash. In marked contrast, Ad5F_{HI}FLAG particles efficiently bound to the M2-affinity gel, since viral DNA was present primarily in the FLAG peptide eluate. Thus, establishing that the heterologous ligand sequence engineered into the HI loop of the knob domain of the fiber incorporated in the intact Ad5 virion remains accessible for interaction with the relevant receptor structure, thereby providing the rationale for the generation of genetically targeted adenovirus vectors on this basis.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention.

While the foregoing description was directed to the preparation of some particular materials, it will be understood that in view of the teaching presented herein, one of skill in the art could readily prepare a variety of modified viral materials, all of which are within the scope of the present invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: David T. Curiel

(ii) TITLE OF INVENTION: METHOD FOR THE PURIFICATION OF VIRUS

(iii) NUMBER OF SEQUENCES: 8

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- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM
- (C) OPERATING SYSTEM: Windows 95
- (D) SOFTWARE: WordPerfect 6.1

(vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Ellen S. Cogen
- (B) REGISTRATION NUMBER: 38,109
- (C) REFERENCE/DOCKET NUMBER: UAB-11552/22

(ix) TELECOMMUNICATION INFORMATION:
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- (B) TELEFAX: 248-647-5210

(2) INFORMATION FOR SEQ ID NO. 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

TAAGGATCCG GTGCCATTAC AGTAGGAAAC AAAAATAA 38

(3) INFORMATION FOR SEQ ID NO. 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:

(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

CATAGAGTAT GCAGATATCG TTAGTGTTAC AGGTTTAGTT TTG 42

(4) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

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(6) INFORMATION FOR SEQ ID NO. 5:

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(A) LENGTH: 62
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5:

TACACTAAAC GGTACCCAGG AAACAGGAGA CACAACTGAC
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(7) INFORMATION FOR SEQ ID NO. 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 6:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 7:

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(9) INFORMATION FOR SEQ ID NO. 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 8:

CGCGCTTGTG ATGGTGATGG TGATGCATG 29

Claims

1. A method for purifying a gene therapy vector polypeptide, comprising the steps of:

providing a vector capsid protein having a heterologous peptide sequence expressed therein;

providing a reagent capable of selectively binding the heterologous peptide sequence;

contacting said vector capsid protein with said reagent, so that said reagent binds to said vector capsid protein; and

releasing said protein from said reagent.

2. The method as in claim 1, wherein the sequence functions to ablate tropism of a homologous fiber protein parent of said protein.

3. The method as in claim 1, wherein said protein is a capsid constituent of a protein coated gene therapy vector, said vector having a capsid surface.

4. The method as in claim 3, wherein said sequence is exposed on the capsid surface.

5. The method as in claim 4, wherein said vector is an adenovirus.

6. The method as in claim 5, wherein said vector is a recombinant adenovirus.

7. The method as in claim 3, wherein the sequence is present at a carboxy terminus of said capsid constituent.

8. The method as in claim 4, wherein the sequence is present in a knob domain of said protein.

9. The method as in claim 8, wherein the sequence is present in a H-I loop of said knob domain.

10. The method as in claim 3, wherein the sequence provides for said protein to interact with other capsid constituents of said vector in a manner comparable with interactions of said homologous fiber protein parent.

11. The method as in claim 1, wherein said reagent is a monoclonal antibody.

12. The method as in claim 1, wherein said reagent is immobilized within a matrix.

13. The method as in claim 1, wherein contacting said protein with said reagent occurs within an affinity chromatography column.

14. The method as in claim 1, wherein contacting said protein with said reagent occurs at atmospheric pressure.

15. The method as in claim 1, wherein releasing said protein occurs in response to the presence of imadozole.

16. A method for purifying an adenoviral vector, comprising the steps of:

providing an adenoviral vector having a capsid, wherein the capsid comprises: a protein having a heterologous peptide sequence therein, the sequence exposed on a surface of the capsid;

providing a reagent capable of selectively binding to the heterologous peptide sequence;

contacting said vector with said reagent, so that said reagent binds to said vector; and

releasing said vector from said reagent.

17. The method as in claim 16, wherein the sequence allows said protein to interact with the capsid so as to yield an identical quaternary structure for said protein, as compared to a homologous protein fiber parent of said protein.

18. The method as in claim 16, wherein the sequence functions to ablate tropism of said homologous protein parent.

19. The method as in claim 16, wherein the sequence is present at a carboxy terminus of said protein.

20. The method as in claim 16, wherein the sequence is present in a knob domain of said protein.

21. The method as in claim 20, wherein the sequence is present in a H-I loop of the knob domain.

22. A purified, immobilized gene therapy vector comprising:

a protein coated vector comprising: a nucleic acid sequence foreign to said vector; a capsid having a surface, said capsid incorporating a protein; and said protein having a heterologous polypeptide sequence therein, wherein the sequence is exposed on the capsid surface so as to yield an identical quaternary structure for said protein, as compared to a homologous protein fiber parent of said protein;

an antibody bound to the heterologous polypeptide sequence; and

a matrix conjugated to said antibody.

23. The vector of claim 22, wherein said vector is adenovirus.

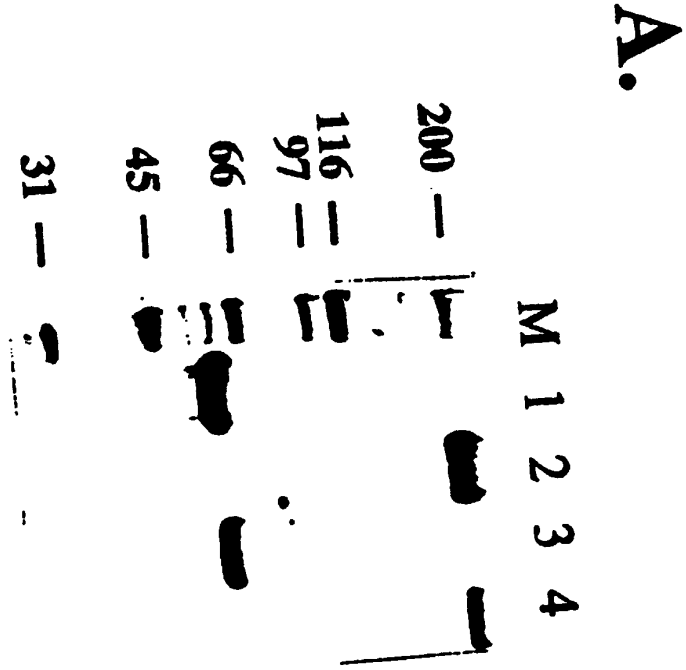
24. The vector of claim 22, wherein the sequence is present at a carboxy terminus of said protein.

25. The vector of claim 22, wherein the sequence is present at a knob domain of said protein.

26. The vector of claim 25, wherein the sequence is present at a H-I loop of the knob domain.

H-strand
HI-loop
I-strand
TKPVTLTIILNGTQETGDTTPSAYSMSFSWDWSGHNYIN
PCR mutagenesis
wild type fiber
truncated H-strand
deleted HI-loop
I-strand
TKPVTLTIISAYSMSFSWDWSGHNYIN
insertion of FLAG sequence
deleted fiber
H-strand
modified HI-loop
I-strand
TKPVTLTIILNGTQETGDTTDYKDDDDKPSAYSMSFSWDWSGHNYIN
FLAG peptide
fiber-FLAG

Figure #1



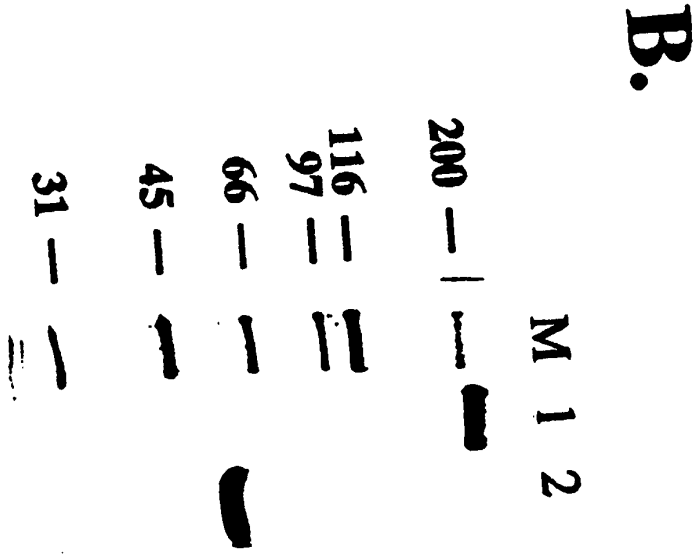


Figure #2

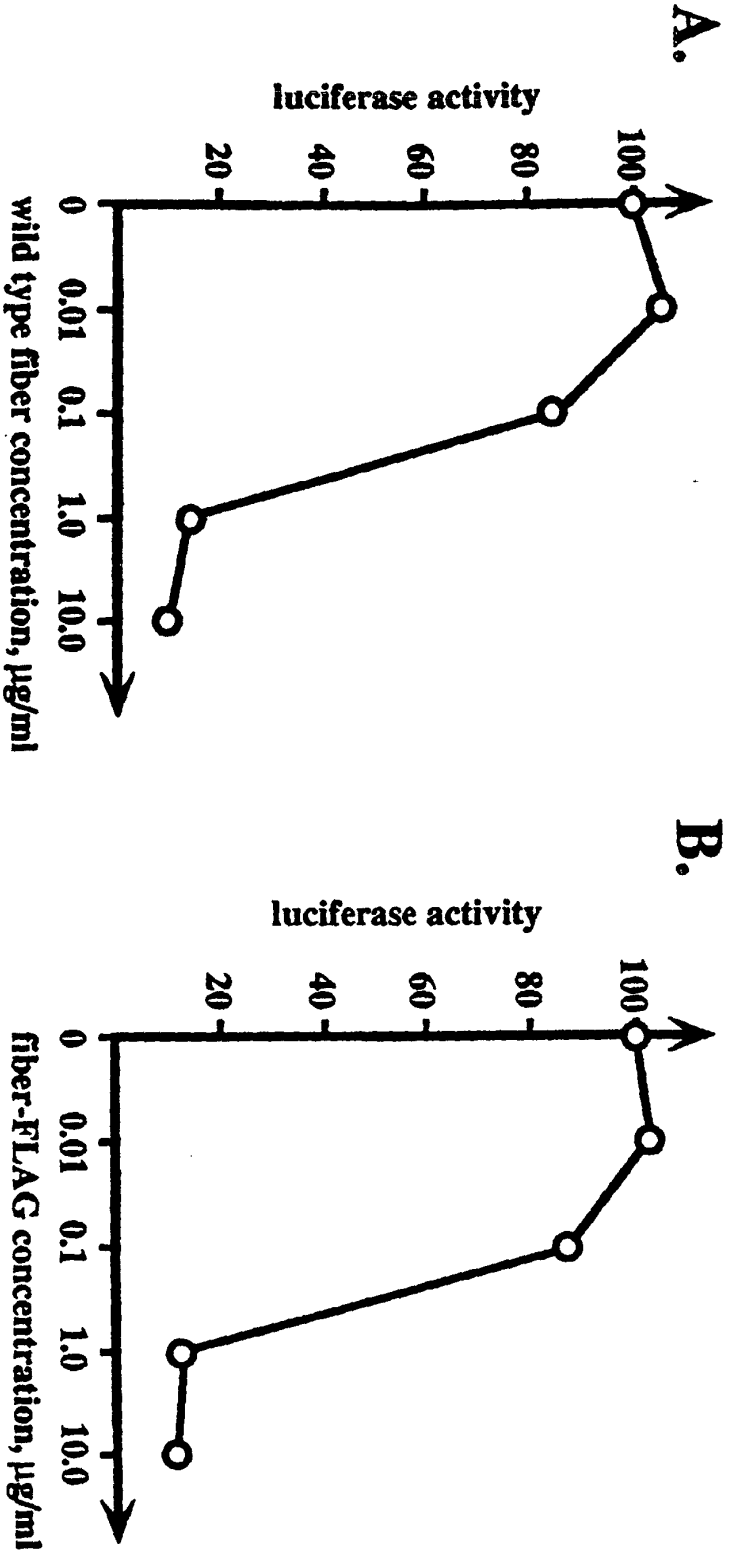

A.
luciferase activity
20
40
60
80
100
0
0.01
0.1
1.0
10.0
wild type fiber concentration, µg/ml
B.
luciferase activity
20
40
60
80
100
0
0.01
0.1
1.0
10.0
fiber-FLAG concentration, µg/ml

Figure #3

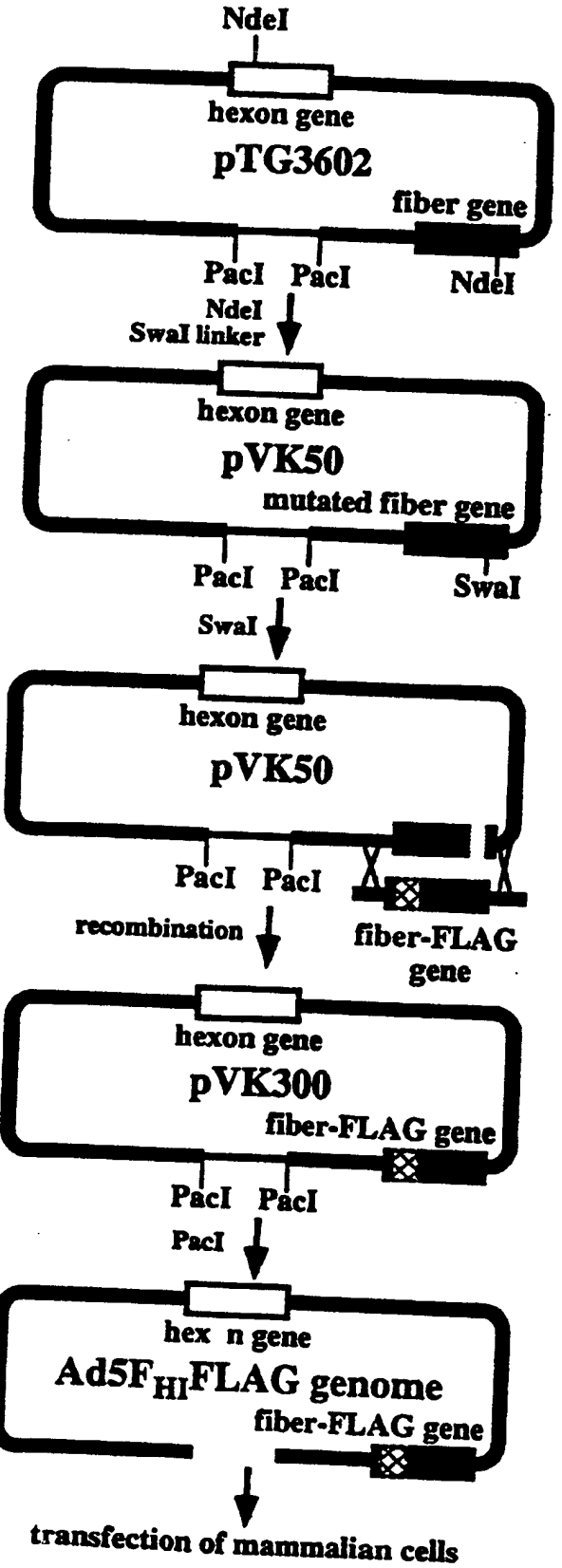
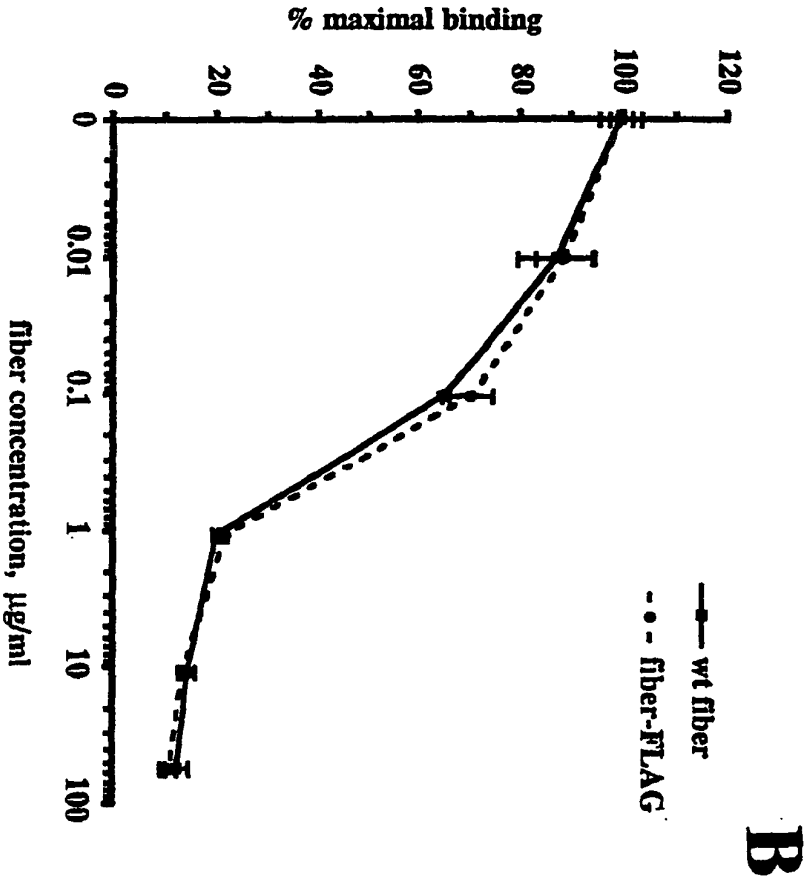

NdeI
hexon gene
pTG3602
fiber gene
PacI PacI
NdeI
NdeI
SwaI linker
hexon gene
pVK50
mutated fiber gene
PacI PacI
SwaI
SwaI
hexon gene
pVK50
PacI PacI
recombination
fiber-FLAG gene
hexon gene
pVK300
fiber-FLAG gene
PacI PacI
PacI
hex n gene
Ad5F$_{HI}$FLAG genome
fiber-FLAG gene
transfection of mammalian cells

Figure #4


% maximal binding
0
20
40
60
80
100
120
0
0.01
0.1
1
10
100
fiber concentration, µg/ml
wt fiber
fiber-FLAG
B

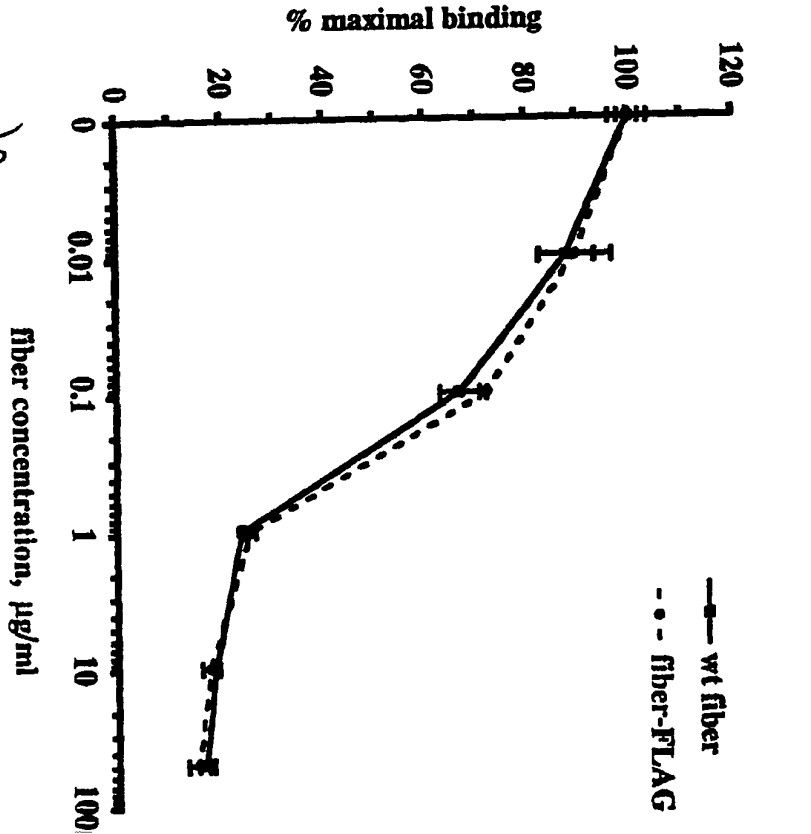

% maximal binding
0
20
40
60
80
100
120
0
0.01
0.1
1
10
100
fiber concentration, µg/ml
wt fiber
fiber-FLAG

Figure #5

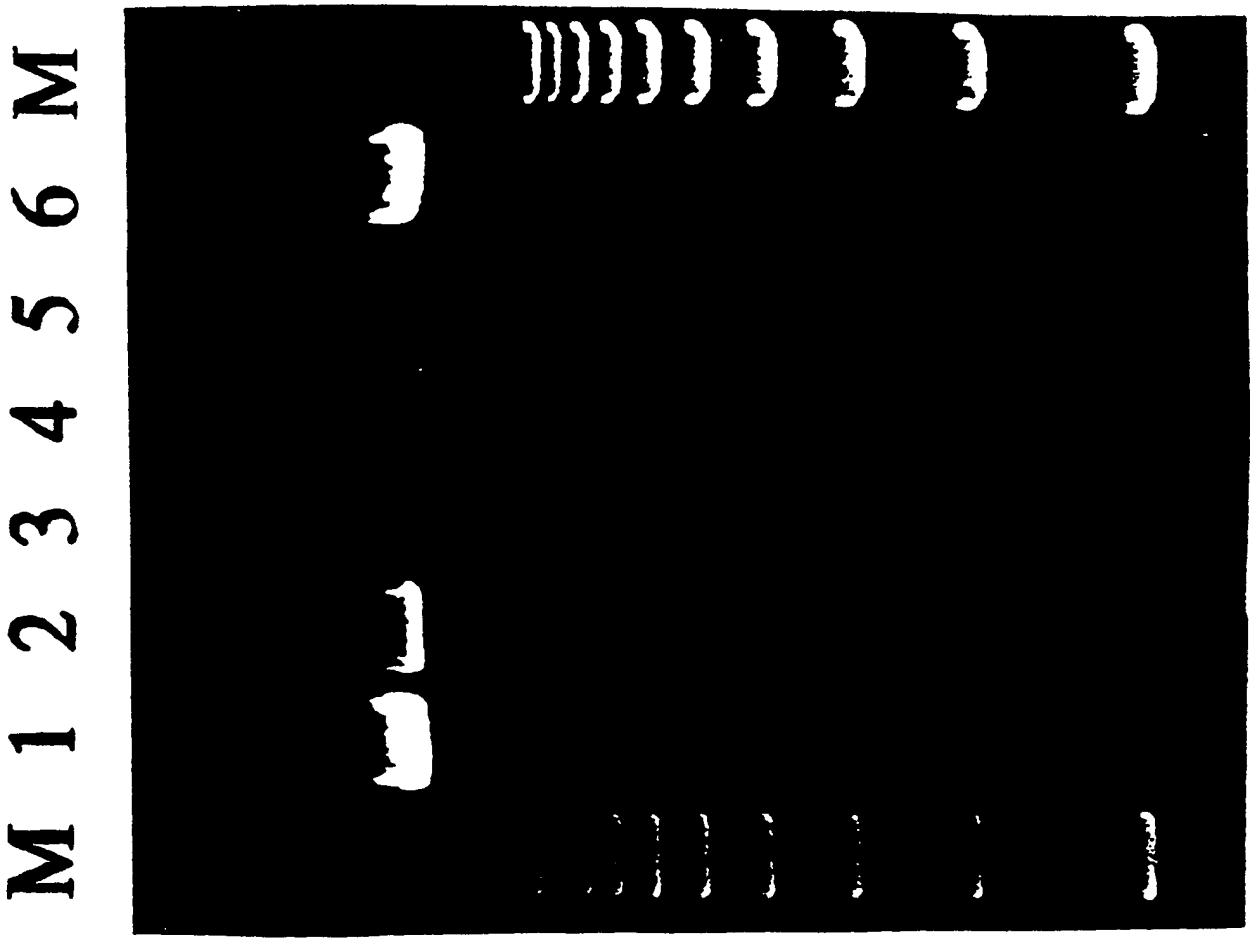


Figure #6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03879

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 7/00, 7/02
US CL :435/235.1, 239

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1, 239

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GALL et al. Adenovirus type 5 and 7 capsid chimera: Fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. J. of Virology. April 1996, Vol. 70, No. 4, pages 2116-2123, see entire document.	1-26
X, P	KRASNYKH, et al. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. J. of Virology. March 1998, Vol. 72, No. 3, pages 1844-1852, see entire document.	1-26
Y, P	WICKHAM et al. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. J. of Virology. November 1997, Vol. 71, No. 11, pages 8221-8229, see entire document.	1-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 MAY 1998	Date of mailing of the international search report 22 JUN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer IREM YUCEL Telephone No. (703) 308-0916

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03879

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	STEVENSON et al. Selective targeting of human cells by a chimeric adenovirus vector containing a modified fiber protein. J. of Virology. June 1997, Vol. 71, No. 6, pages 4782-4790, see entire document.	1-26
Y	WHEATLEY, J.B. Multiple ligand applications in high-performance immunoaffinity chromatography. J. of Chromatography. 1992, Vol. 603, pages 273-278, see entire document.	1-26
Y	STEVENSON et al. Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. J. of Virology. May 1995, Vol. 69, No. 5, pages 2850-2857, see entire document.	1-26
Y	KRASNYKH et al. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. J. of Virology. October 1996, Vol. 70, No. 10, pages 6839-6846, see entire document.	1-26
Y	WICKHAM et al. Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. J. of Virology. October 1996, Vol. 70, No. 10, pages 6831-6838, see entire document.	1-26
Y	WO 95/26412 A1 (THE UAB RESEARCH FOUNDATION) 05 October 1995, see entire document.	1-26
Y	WO 96/26281 A1 (GENEVEC, INC.) 29 August 1996, see entire document.	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03879

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG (MEDLINE, BIOSIS, SCISEARCH), STN (CAPLUS)

search terms: inventor's names, vir? purif?, isolat?, protein?, chromatograph?, affinity? tropism, adenovir?, fiber or fibre, vector?